REVIEW

The origin recognition complex: from simple origins to complex functions

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Eukaryotic DNA replication is an intricate process that requires the coordinate and tightly regulated action of numerous molecular machines. Eukaryotic cells must choose hundreds if not thousands of origins of replication and assemble multiple proteins or protein complexes at these sites. During S phase, these complexes are activated in a reproducible, temporal program to generate twice this number of replication fork machines dedicated to DNA synthesis. Moreover, these machines must overcome the highly packaged nature of the eukaryotic genome to access all DNA sites, without exception.

The key factor in the selection of initiation sites and the assembly of the replication machinery is the initiator protein. Originally proposed by Brenner, Cuzin, and Jacob based on genetic findings, the replicon model hypothesized the existence of two essential components required for replication initiation: the initiator and the replicator (Jacob et al. 1963). The initiator is a positively acting factor that recognizes a specific DNA sequence within the genetic element required for initiation, called the replicator. The replicator usually overlaps the site of replication initiation, the origin of replication. Subsequent studies in *Escherichia coli*, other bacteria, and many bacteriophages and eukaryotic viruses identified initiator proteins and confirmed this basic model. Unlike these organisms, each of which uses a single replicator to duplicate their genome, eukaryotic chromosomes each require multiple replicators to ensure complete replication of their genome in a timely manner. Although it was possible that multiple initiators functioned in this more complex situation, studies over the last 10 years have demonstrated that a single conserved eukaryotic initiator directs all of these initiation events: the origin recognition complex (ORC).

The well-defined replicators of *Saccharomyces cerevisiae* provided the critical tools to identify ORC. These sequences were originally identified by their ability to confer stable propagation to yeast episomes and were called autonomous replicating sequences (ARSs). Subsequent studies provided definitive evidence that these

sites represented true chromosomal replicators and overlapped with their associated origins of replication (for review, see Bell 1995). Mutational analyses of ARS elements identified an essential, conserved, 11-bp element associated with all ARS elements known as the ARS consensus sequence (ACS or "A element"), as well as multiple 10–15-bp "B-elements" that also contribute to origin function. The conserved nature of the ACS made it a likely target for a eukaryotic initiator and many efforts were made to identify such a factor. ORC was ultimately identified based on its affinity for the ACS and was therefore a strong candidate for the eukaryotic initiator (Bell and Stillman 1992). DNA binding by this six-protein complex (referred to as Orc1p–Orc6p, from largest to smallest subunit) requires ATP (see below), and it is likely that this requirement as well as the low-abundance of ORC thwarted previous attempts to identify this factor.

Abundant evidence now supports the conclusion that ORC is the *S. cerevisiae* initiator. Point mutations in the ACS that eliminate ORC binding in vitro and in vivo also result in loss of replicator activity supporting the hypothesis that ORC mediates the function of this critical element (Bell and Stillman 1992; Li and Herskowitz 1993; Aparicio et al. 1997; Tanaka et al. 1997). Mutations in the genes encoding the subunits of the ORC complex result in DNA replication defects and prevent the association of all other replication factors at the origin (Bell et al. 1993; Foss et al. 1993; Micklem et al. 1993; Santocanale and Diffley 1996; Dillin and Rine 1997, 1998). Finally, in vivo footprinting, protein–DNA cross-linking, and chromatin precipitation studies all indicate that ORC is bound to *S. cerevisiae* origins of replication throughout most if not all of the cell cycle (Diffley et al. 1994; Aparicio et al. 1997; Liang and Stillman 1997; Tanaka et al. 1997).

Unlike the simple replicators of *S. cerevisiae*, replicators in other eukaryotic organisms remain poorly defined (for review, see Bielinsky and Gerbi 2001; Gilbert 2001). The most obvious difference between replicators in *S. cerevisiae* and other organisms is at the level of size. Even in the single cell eukaryote, *Schizosaccharomyces pombe*, replicators are found to be 5- to 10-fold larger than their *S. cerevisiae* counterparts. Replicators derived from multicellular eukaryotes are still less well defined

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and only a few have been mapped in any detail. In multicellular organisms the number and location of origins are regulated during development. Particularly dramatic examples of such changes in origin usage are observed during development of *Drosophila melanogaster* and *Xenopus laevis*. The rapid DNA replication observed in the early embryos of these species is mediated by tens of thousands of origins spaced by as little as 4–7 kb (Blumenthal et al. 1974). Later in development, inter-origin distances increase, reducing the number of replicators used by ≥ 10 -fold (Hyrien et al. 1995; Sasaki et al. 1999). Interestingly, in vitro experiments using *Xenopus* egg extracts indicate that there is little or no sequence specificity required for the initiation of DNA replication (Coverley and Laskey 1994). Indeed, although numerous studies have pursued the identification of essential elements within other replicators, as of yet, no element with similar properties to the *S. cerevisiae* ACS has been identified in any other eukaryote.

Despite differences in their structure and a lack of obvious conserved sequences among eukaryotic replicators, ORC is a conserved feature of replication initiation in all eukaryotes tested. ORC-related genes have been identified in organisms ranging from *S. pombe* to plants to humans (for review, see Dutta and Bell 1997). Mutational analysis of ORC-related genes in *S. pombe* and *D. melanogaster* both reveal defects in DNA replication (Muzy-Falconi and Kelly 1995; Grallert and Nurse 1996; Landis et al. 1997; Pinto et al. 1999; Loupart et al. 2000; Pflumm and Botchan 2001). Similarly, immunodepletion studies using *X. laevis* replication competent extracts indicate an absolute requirement for ORC to initiate DNA replication (Carpenter et al. 1996; Romanowski et al. 1996; Rowles et al. 1996). Although no genetic evidence indicates that ORC functions in human chromosomal DNA replication, plasmids replicating in human cells by virtue of an Epstein Barr Virus (EBV) replicator are defective in cells carrying an *ORC2* mutation (Dhar et al. 2001b). This, together with other evidence, indicates that EBV uses the human replication initiation machinery, and suggests that HsORC functions in human DNA replication. Thus, even in multicellular organisms that have yet to show evidence of conserved sequences required for replication initiation, ORC has been retained as an essential component of the replication initiation process.

In all cases studied, ORC binding to the replicator is the first step in the establishment of a multifactor assembly called the prereplicative complex (pre-RC, for review, see Bell and Dutta 2002). After ORC binds DNA, it recruits several additional DNA replication factors including Cdc6p, Cdt1p, and the Mcm2–7p complex (MCM complex) to the origin. Pre-RC assembly is initiated at the M/G₁ transition of the cell cycle and, once established, the pre-RC is maintained during G₁. On entry into S phase, existing pre-RCs are activated in a characteristic temporal pattern as cells progress through S phase. Both cyclin-dependent kinases (CDKs) and the Dbf4p-dependent kinase (DDK, whose catalytic subunit is Cdc7p) are required to trigger initiation, although the

exact targets and consequences of these modifications remain unclear. In addition to activating existing pre-RCs, the elevation of CDK activity also plays a key role in preventing new pre-RC formation until the following S phase (for review, see Bell and Dutta 2002). In this review, I will discuss first what we know about the role of ORC in each of these events. Then, I will describe studies addressing other roles for ORC in the cell. When relevant, ORC derived from different organisms will be distinguished using the initials of the genus and species names preceding ORC (e.g., SpORC is *S. pombe* ORC).

ORC and origin specification

Studies of ORC across a wide range of eukaryotic species indicate that localization of this complex is the initial step in determining the sites of DNA replication initiation. Despite this, the mechanism by which ORC is localized to origins of replication remains incompletely understood. In *S. cerevisiae*, the ACS and B1 elements play a large part in directing ORC to origins of replication, however, these sequences are unlikely to be sufficient to direct ORC localization independently. In other eukaryotes, sequences that direct ORC binding are unknown. Indeed, in some cases there appears to be little or no DNA sequence selectivity for the location of origins.

DNA binding by ScORC

The most well characterized activity of ORC is its ability to bind origin DNA. Although initial studies identified the ACS as the *S. cerevisiae* ORC (ScORC) binding site (Bell and Stillman 1992), further studies demonstrated that there was at least one additional interaction within the adjacent B1 domain (Rao and Stillman 1995; Rowley et al. 1995). Detailed studies of the contacts between ORC and origin DNA support an extensive interaction between ScORC and the origin and identify additional sequences that interact with ORC between the A and B1 region (Lee and Bell 1997). Interestingly, these studies also found that ORC primarily interacts with the A-rich strand of the ACS, suggesting that other replication factors can gain access to the opposite strand of the ACS without displacing ORC.

The interaction of ORC with origin DNA involves multiple subunits. Protein–DNA cross-linking studies indicate that four of the six ScORC subunits (Orc1p, Orc2p, Orc4p, and Orc5p) form close contacts with the origin DNA (Fig. 1A; Lee and Bell 1997). Although ScOrc3p does not cross-link to origin DNA, this subunit is required for ScORC to bind origin DNA, suggesting an involvement in positioning the other ORC subunits for appropriate interaction with the origin. Only ScOrc6p is dispensable for sequence-specific DNA binding by ScORC, although it is still required for ORC function in vivo (Lee and Bell 1997). In contrast, studies of *Drosophila* ORC indicate that all six ORC subunits are required for efficient DNA binding as well as for in vitro DNA replication (Fig. 1C; Chesnokov et al. 2001).

S. cerevisiae *S. pombe* *D. melanogaster*

Figure 1. Association of origin recognition complex (ORC) with known replicators. The association of ORC with *Saccharomyces cerevisiae* origins involves four of the six subunits (Orc1p, Orc2p, Orc4p, and Orc5p) recognizing the 11-bp autonomous replicating sequence (ARS) consensus sequence (ACS) as well as several less well-defined adjacent DNA sequences including a subset of B1 element sequences. Binding of *Schizosaccharomyces pombe* ORC (SpORC) to its cognate origins involves a clearly distinct mechanism involving nine repeats of an AT-hook motif found uniquely at the N-terminus of the *S. pombe* Orc4 subunit. A discrete binding site has not been identified, however recent studies indicate that SpORC recognizes stretches of A-rich DNA. Less is known about the sequences recognized by *Drosophila* ORC (DmORC), however, ATP-dependent binding to both the ACE3 and ori- β elements of the third chromosome have been detected in vivo and in vitro. Unlike ScORC, all six subunits of DmORC are required for DNA binding. See text for additional details and references.

The complex nature of the ORC–DNA interface has interfered with a precise determination of the DNA binding motifs involved in origin recognition. With one exception (see below), sequence comparison has not identified any clear DNA binding motifs in any ORC subunit from any species. The determination of the crystal structure of an archaeal Cdc6p/Orc1p-related protein has revealed a candidate DNA binding domain (Liu et al. 2000). Analysis of this structure revealed a region of the C-terminus of the archaeal protein that forms a fold related to the winged-helix DNA binding domain. More importantly, this region is conserved in both Cdc6p and Orc1p derived from multiple species, raising the interesting possibility that this region of Orc1p mediates, at least in part, DNA binding by ORC.

Recent work has taken advantage of the ability to cross-link ORC to its binding sites in vivo to identify ORC binding sites across the *S. cerevisiae* genome (Wyrick et al. 2001). This study used the chromatin immunoprecipitation (ChIP) technique to isolate DNA fragments cross-linked to ORC and applied the isolated

DNA to DNA microarrays to identify the associated DNAs. Approximately 460 ORC binding sites were identified in G₂/M arrested cells (cells arrested at G₂/M were chosen due to the occlusion of ORC in G₁ cells by other members of the pre-RC, making detection of ORC by ChIP difficult; Aparicio et al. 1997). Of these binding sites, a majority are within intergenic regions and >90% are in close proximity to the binding sites of the MCM proteins during G₁, suggesting that they direct the formation of pre-RCs. By combining the information of ORC and MCM localization with the sites of known origins of replication, an algorithm was developed to predict sites of potential origins. Using this algorithm, 429 proposed ARSs were predicted. When a subset of these sites were tested directly, ~80% acted as ARS elements in plasmids. In addition, 13 of 15 sites predicted to act as origins on *S. cerevisiae* chromosome X by an alternative whole genome analysis of replication intermediates were included in this set (Raghuraman et al. 2001). Although not a perfect predictor of origin activity, this approach shows a robust ability to delimit the sites of possible origins to within <1 kb. When combined with other methods to confirm the sites of replication initiation, this method has the potential to greatly increase the number of identified origins in other eukaryotes. It is interesting to note that there appear to be more predicted ORC binding sites than origins, suggesting that a subset of these sites are involved in other cellular functions (see below). In addition, ORC does not interact strongly with inefficient origins in G₂/M arrested cells, although it is likely that other factors stimulate ORC binding to these sites during G₁ (see below; Wyrick et al. 2001).

Can ORC bind specific sequences in other eukaryotes?

Although a consensus ORC binding site has not been identified other than the *S. cerevisiae* ACS, studies in several other organisms are consistent with ORC recognizing DNA sequences within known replicators in vivo. ChIP experiments using *S. pombe* cells have demonstrated that ORC is associated with multiple origins in vivo (Ogawa et al. 1999). In situ analysis of DmORC using mitotic and polytene chromosomes shows a staining pattern consistent with a defined pattern of DmORC localization along the chromosomes (Pak et al. 1997). ChIP experiments confirm the association of DmORC with elements of the well-characterized ACE3 origin of DNA amplification. These studies also suggest that DmORC interacts with multiple sequences adjacent to ACE3 in a manner that does not appear to be highly sequence specific but nonetheless requires proximal ACE3 DNA (Austin et al. 1999). The specificity and biological relevance of these additional interactions remains unclear. Similar cross-linking studies indicate human ORC (HsORC) associates with the EBV origin of replication, although association with this site appears to be mediated by the viral DNA binding factor, EBNA1 (Chaudhuri et al. 2001; Dhar et al. 2001b; Schepers et al. 2001).

Unlike other ORC analogs, SpORC includes at least

one clear DNA binding motif (Fig. 1B). Cloning of the gene encoding SpOrc4p, revealed that this subunit of SpORC contains nine repeats of an AT-hook motif not present in the Orc4 subunits derived from other species (Chuang and Kelly 1999). This motif was previously identified as a DNA binding domain in HMG proteins that recognizes AT-rich tracts of DNA (Reeves and Beckerbauer 2001). Interestingly, studies of other AT-hook proteins suggest that they can recognize or induce structural changes in bound DNA. In vitro binding experiments indicate that the SpOrc4p can interact with both the *ars3002* and *ars1* origins of replication (Chuang and Kelly 1999; Kong and DePamphilis 2001; Lee et al. 2001). DNase I footprinting identified several specific AT-rich binding sites within these origins, most of which included regions of poly(A) (≥ 5 consecutive A residues; Kong and DePamphilis 2001; Lee et al. 2001). Such binding is not simply association with AT-rich DNA because other sequences that included stretches of poly(AT) were not bound. Interestingly, although the remaining five subunits of SpORC were able to interact with SpOrc4p bound to DNA, they did not appear to have any sequence-specific DNA binding activity on their own, nor did they alter the interaction of prebound SpOrc4p with the origin DNA (Kong and DePamphilis 2001; Lee et al. 2001). These findings demonstrate that SpOrc4p is a sequence-specific DNA binding protein and indicate that SpOrc4p is largely responsible for the sequence specificity of SpORC.

Studies of metazoan ORCs have been less clear concerning specific ORC binding sites. Studies of replication in embryonically derived extracts indicate that virtually any sequence can be replicated (for review, see Coverley and Laskey 1994). Despite this apparent lack of specificity, studies of *Xenopus* egg extracts indicate that XIORC is limiting for DNA replication and saturates added DNA at approximately one ORC molecule per 16 kb (Rowles et al. 1999; Blow et al. 2001). Saturation could be attributable to a limited number of specific DNA binding sites, limited access to DNA, or stabilization of a subset of bound ORC molecules by other replication or chromatin factors.

Of the ORCs derived from multicellular organisms, only DmORC has been extensively studied in direct DNA binding experiments (Fig. 1C). Gel shift assays using DmORC purified from early embryos found a clear preference for binding to critical elements of the *Drosophila* chorion amplification loci (*ACE3* and *ori- β /AER-d*) but significantly lower affinity for two adjacent sequences with the same AT-content (Austin et al. 1999). Recombinant DmORC also binds *ori- β* and *ACE3* but this affinity is not reported to be any higher than to equally AT-rich sequences such as the ends of P-elements (Chesnokov et al. 1999, 2001), suggesting that there may be differences in the specificity of extract-derived and recombinant DmORC. Interestingly, the same recombinant DmORC specifically binds the amplification origin II/9A of the fly, *Sciara coprophila* at a sequence near the site of initial nucleotide incorporation (Bielinsky et al. 2001). This same region is also associ-

ated with *Sciara* ORC in vivo. Despite the differences in the degree of specificity in these reports, in all cases sequence-specific DNA binding by DmORC is ATP-dependent. Overall, these studies suggest that ORC from metazoans retains DNA sequence specificity but apparently not at the level observed for ORC derived from *S. cerevisiae*.

It is important to note that although no distinct binding site for ORC has been identified in organisms other than *S. cerevisiae*, this may be attributable to a lack of tools to identify such a site. Only recently has ORC been purified from species other than *S. cerevisiae* and few studies of the DNA binding properties of these complexes, either in vivo or in vitro, have been performed. Interestingly, for the two species in which such studies have been performed (*S. pombe* and *Drosophila*) there is clear evidence for specific DNA binding activity. In addition, the lack of robust assays to identify critical sequences within metazoan origins has hampered the identification of potential ORC binding sites.

Beyond DNA sequence specificity: helping ORC to the origin

Even in *S. cerevisiae* it is unlikely that the sequence specificity of ORC is sufficient for localization to origin sequences. Searching for matches to the ACS in the yeast genome indicates that this consensus alone is not sufficient to identify an ORC binding site or an origin (Newlon and Theis 1993; Wyrick et al. 2001). Mapping of ScORC binding sites across the yeast genome supports a hypothesis that there are other factors that facilitate ScORC localization. These studies find that strong ScORC binding sites are distributed throughout the genome with an average spacing of ~20–30 kb (Wyrick et al. 2001). However, in vitro DNA binding experiments suggest that ScORC does not have the intrinsic DNA binding specificity required to recognize a single sequence every 20–30-kb region (R.D. Klemm, J.R. Lipford, and S.P. Bell, unpubl.).

If ORC does not have the ability to select origins based solely on its own affinity for specific DNA sequences, then how does it become localized across the genome? There are three general types of mechanisms that could be involved (Fig. 2). First, the association of other replication factors with ORC could enhance its interaction with appropriate sequences (Fig. 2A). Second, other factors that interact specifically with DNA could be involved in recruiting ORC to specific sites in the genome (Fig. 2B). Third, attributes of chromatin structure in specific regions of the genome could restrict or delineate areas that ORC can function (Fig. 2C). There is evidence consistent with each of these possibilities.

Studies of the interaction of ORC with origin DNA in the presence of other pre-RC components suggest that these factors may contribute to the specificity of ORC localization (Fig. 2A). One candidate to increase the specificity of ORC association with DNA is Cdc6p. Studies of the association between ScORC and ScCdc6p indicate that it is facilitated by origin DNA (Mizushima

Figure 2. Potential mechanism controlling origin recognition complex (ORC) localization. Several possible mechanisms are likely to contribute to the specification of ORC chromosomal binding sites. (A) In *Saccharomyces cerevisiae* cells, a key component of localization is the recognition of specific sequences within the replicator. Additional replication factors (e.g., Cdc6p), however, may augment ORC DNA binding. (B) Studies of *Drosophila* and Epstein Barr Virus replication suggest distinct, sequence-specific DNA binding factors direct ORC localization through protein-protein interactions. (C) The localization of origins to intergenic regions suggests that local chromatin structure or the negative action of transcription may serve to restrict and/or facilitate ORC localization. Other mechanisms that would fall into this class would be displacement by RNA polymerase, modification of DNA, and modification of histones. See text for details and references.

et al. 2000). Moreover, the interaction between ORC and Cdc6p reduced the off-rate of ORC from origin but not from nonorigin fragments. Although the mechanism responsible for the reduced off-rate is unclear, it required an intact Cdc6p ATP-binding motif. In addition, the interaction between ORC and Cdc6p appeared to alter the conformation of one or more ORC subunits and this change could lead to enhanced interaction with the origin (Mizushima et al. 2000).

A role for sequence-specific DNA binding factors in the recruitment of ORC to specific genomic loci has been suggested by studies of the *Drosophila* *ACE3* amplification origin (Fig. 2B). The *Drosophila* analogs of the E2F1 transcription factor (dE2F1) and Rb (Rbf) are associated with a subset of ORC in *Drosophila* cells (Bosco et al. 2001). More importantly, mutant alleles of dE2F1, Rbf, and the *Drosophila* Dp analog each have strong effects on the localization of ORC and the function of the *ACE3* amplification origin (Royzman et al. 1999; Bosco et al. 2001). Studies of the association of HsORC with the EBV origin of replication suggest that the viral transcription factor EBNA1 could play a similar role in the recruitment of ORC to these sites (Chaudhuri et al. 2001; Dhar et al. 2001b; Schepers et al. 2001). It is interesting to note that, if such a mechanism does operate in eukaryotic cells, then it is likely that a number of different DNA binding factors would be involved in recruiting ORC. Otherwise, origins would each contain a consensus-binding site for the ORC recruiting factor.

Other mechanisms that could influence ORC localization include local or global chromatin structure, DNA modification, or transcription itself. There is a strong tendency for origins and ORC binding sites to be located in intergenic regions (Brewer 1994; Gilbert 2001; Wyrick et al. 2001), raising the possibility that ORC could be displaced by elongating RNA polymerases. Studies in *S. cerevisiae* argue against such a mechanism, as origins and ORC binding sites appear restricted to intergenic regions even in poorly transcribed regions of the genome.

Alternatively, the open chromatin structure associated with the control regions of genes may provide increased access for ORC or other replication factors (Fig. 2C). Consistent with nucleosomes restricting possible sites of ORC localization, *in vivo* studies indicate that positioning a nucleosome over the ORC binding site at an origin inactivates origin function (Simpson 1990). More importantly, even subtle changes in the local chromatin structure can have a significant impact on origin usage, although this effect does not appear to be at the level of ORC DNA binding (Lipford and Bell 2001). Such a mechanism could explain the increased restriction of origin sites as embryonic cells begin transcription (Hyrien et al. 1995; Sasaki et al. 1999). Modification of DNA and/or histones also represents a likely mechanism to restrict the number of possible ORC binding sites. In this regard, it is interesting that human Orc1p is found to associate with the Hbo1 histone acetyltransferase (Iizuka and Stillman 1999; Burke et al. 2001). Finally, although neither ORC binding sites nor origins of replication colocalize with sites of chromatid cohesion in *S. cerevisiae* (Blat and Kleckner 1999; Laloraya et al. 2000; Wyrick et al. 2001), it is possible that proteins involved in higher order packaging of the genome influence ORC localization either directly or indirectly.

It is likely that some composite between ORC DNA sequence specificity and these other methods to enhance or restrict ORC DNA binding will be at work in all organisms. Each organism is likely to balance this equation in a characteristic manner to accommodate different requirements for origin density and location. Indeed, it is likely that similar mechanisms are involved in the localization of other DNA binding factors in the cell. For example, it is possible that there are general methods used across eukaryotic genomes to prevent sequence-specific binding to transcribed regions. As an understanding of how ORC interacts with DNA emerges, it will be interesting to see if the elements involved in specific binding in *S. cerevisiae* are adapted for other

DNA interactions in organisms showing less sequence specificity. Given the clear conservation of ORC and its architecture across eukaryotes, it is unlikely that all of the DNA interaction domains have been eliminated. Given that four of the six ORC subunits contact the DNA in ScORC, it is possible that one or more of these contacts are involved in interactions with the origin required for pre-RC formation or some other nonsequence-specific event. Consistent with this hypothesis, despite the apparently unique method of DNA binding for SpORC, the SpOrc1–5 subunits are still well conserved.

Regardless of the mechanism that is used to localize ORC and other factors to origins of replication, these proteins are clearly excellent markers for potential origins of replication. The development of technology to identify sites of DNA binding for ORC and other replication proteins across the genome will provide a powerful tool to map these loci in the future. Indeed, just as the conserved, simple origins of *S. cerevisiae* were critical to the identification of ORC, the identification of ORC and other origin-associated factors represents a key tool to identify and characterize their cognate origins/binding sites.

Pre-RC assembly and the ATP regulation of ORC

Once bound to origin DNA, ORC recruits the components of the pre-RC. This involves the interaction of ORC with at least two and possibly several other components of the preinitiation machinery. In addition, ORC remains at the origin throughout G₁ and S phase, raising the possibility that ORC functions in events after pre-RC formation. Interestingly, recent studies suggest that these functions are unlikely to involve the recruitment of other replication factors. The identification of ORC as an ATPase has led to the hypothesis that its function in these events is regulated by nucleotide.

ATP regulation of ORC function

ORC generally requires ATP to interact specifically with origin DNA (the exception being SpORC) and in all species studied, Orc1p, Orc4p, and Orc5p, each contain potential ATP binding sites based on amino acid sequence analysis. Mutations in the ScOrc1p and ScOrc5p ATP binding motifs both interfere with ScORC ATP binding (Klemm et al. 1997). Assessment of the consequences of making the same mutations in vivo found that a mutation in the ScOrc1p ATP binding site was lethal whereas mutation of the ScOrc5p ATP binding motif had no obvious phenotype. Consistent with the lethal phenotype of the ScOrc1p mutation, in vitro studies indicate that mutation of the ScOrc1p but not the ScOrc4p or ScOrc5p subunits interfere with DNA binding by ORC (Klemm et al. 1997). DmORC also requires ATP to bind tightly to DNA and includes ATP binding motifs in DmOrc1p, 4p, and 5p (Chesnokov et al. 2001). Analysis of DmORC complexes mutated in these three motifs indicates that only mutations in the DmOrc1p ATP binding motif in-

terfere with DNA binding and ORC function in reconstituted DNA replication. Thus, despite the conservation of the Orc4p and Orc5p ATP binding motifs across multiple species, a requirement for these motifs for ORC function has yet to be established. It remains possible that ATP binding to these domains is required for non-essential ORC functions, such as DNA replication checkpoints or transcriptional silencing (see below).

ORC also hydrolyzes ATP (Klemm et al. 1997; Chesnokov et al. 2001). As with ATP control of DNA binding, studies of ATP hydrolysis by ORC have been performed for both DmORC and ScORC with similar findings. In both cases only mutations in the Orc1p ATP binding motif interfere with ATP hydrolysis. Binding of ScORC to origin DNA inhibits ATP hydrolysis by 8- to 10-fold and a similar twofold inhibition by DNA is observed for DmORC. For ScORC, this effect requires the presence of the ACS. Origin DNA lacking an ORC binding site has little or no effect on ATP hydrolysis by ORC. Thus, Orc1p is a central regulator of ORC function. Orc1p directly interacts with origin DNA, binds the ATP required for ORC to recognize origin DNA, and alters its rate of ATP hydrolysis in response to origin binding.

Together these findings suggest a model in which, once ORC is bound to origin DNA, the bound ATP that is required for stable DNA binding is not immediately hydrolyzed. Instead, hydrolysis of this ATP is proposed to be conserved for a subsequent step in the replication process. There are several key issues that remain to be addressed regarding this model. Is ATP binding by ORC required for additional events beyond origin recognition? What event(s) triggers ATP hydrolysis by ORC? What are the consequences of ATP hydrolysis by ORC? Recent studies have shed some light on these questions.

In addition to binding double-stranded DNA (dsDNA) forms of the origin, ScORC also has a high affinity for single-stranded DNA (ssDNA, $K_d \approx 10^{-8}$; Lee et al. 2000). Unlike ScORC binding to dsDNA, ScORC's association with ssDNA is neither sequence-specific nor ATP-dependent. Instead, the affinity of ScORC for ssDNA is determined by the length of the ssDNA, with molecules greater than ~80 bases having the highest affinity. This association requires the same five subunits of ScORC required for dsDNA binding. Association with ssDNA alters ScORC function in two ways (Lee et al. 2000). First, it stimulates the rate of ATP hydrolysis ~20-fold relative to ORC bound to origin DNA. Second, electron microscopy analysis of ScORC function indicates that binding to ssDNA stabilizes an altered structure. Because ssDNA is a direct consequence of the initiation process, these observations suggest that the generation of ssDNA during initiation is coupled to activation of the ORC ATPase and alteration of its structure.

ORC recruitment of replication factors: ORC as a landing pad

Once bound to DNA, ORC plays a critical role in the recruitment of additional replication factors to the origin. Studies in *S. cerevisiae*, *S. pombe*, and *Xenopus* have

all demonstrated that ORC is required for the recruitment of the known components of the pre-RC to the origin. The recruitment of these factors occurs in an ordered process: Cdc6p and Cdt1p load first followed by Mcm2-7p. As cells pass through the G₁ to S phase transition additional replication factors are recruited to the origin. These factors include CDKs and DDK, Cdc45p, the three eukaryotic DNA polymerases, and the eukaryotic ssDNA binding protein, RPA (for review, see Bell and Dutta 2002). As with the components of the pre-RC, either cells or extracts lacking ORC fail to assemble these additional factors at the origin. Although it is clear that ORC is required to initiate the assembly of all of these factors at the origin, ORC has only been shown to interact directly with a small subset of these factors. Recent studies of replication in *Xenopus* extracts suggest that many of these factors do not require ORC bound to the origin at the time of assembly (Hua and Newport 1998; Rowles et al. 1999). The simplest interpretation of ORC's role in these events is that it acts as a "landing pad" to localize other factors to the site of initiation.

There is abundant evidence that ORC interacts directly with Cdc6p. In vitro studies have identified a direct interaction between ORC and Cdc6p that depends on binding of ORC to origin DNA (Mizushima et al. 2000). In human cells, HsOrc1p and HsCdc6p coelute through multiple columns and can be shown to interact with one another directly in in vitro assays (Saha et al. 1998). This suggests the interesting possibility that these two factors dynamically associate with the remainder of HsORC in a coordinated fashion (see below). Interestingly, several lines of evidence support the hypothesis that this interaction is likely to require ATP-bound Orc1p. First, the requirement for origin DNA to observe the ORC–Cdc6p interaction described above is consistent with a requirement for ATP-bound ORC. Second, using a reconstituted assay for pre-RC formation, ATP was required for association of Cdc6p with ORC and the origin (Seki and Diffley 2000). Third, studies of a dominant allele of *ScORC1* that is defective in ATP hydrolysis suggest that the dominant effect is mediated by excess ATP-bound ORC titrating Cdc6p away from the origin (Klemm and Bell 2001). In addition to direct biochemical studies, genetic interactions between ORC and Cdc6p have also been observed including the ability of excess *CDC6* expression to suppress the lethality of an *Scorc5-1* allele (Liang et al. 1995).

Interaction of ORC with other replication factors is less well documented. Although it is likely that ORC will interact with Cdt1p and Mcm2-7p, evidence for these interactions is largely circumstantial. For example, studies of *Xenopus* replication extracts and *S. pombe* cells indicate that Cdc6p and Cdt1p independently load onto chromatin in an ORC-dependent manner (Maiorano et al. 2000; Nishitani et al. 2000). This would suggest a direct interaction between ORC and Cdt1. In addition, given the relative sizes of ORC and the MCM complex, these complexes are likely to interact during pre-RC formation. Studies of the nuclear localization and association of ScDbf4p (the regulatory subunit of

ScDDK) with chromatin also suggest a direct interaction with ORC (Pasero et al. 1999). Mutations in *ScORC2* interfere with the formation of subnuclear ScDbf4p foci and the association of ScDbf4p with chromatin fractions. Interestingly, this association is independent of Cdc6p and MCM loading suggesting that the requirement for ORC is direct and not attributable to ORC's role in pre-RC formation. This interaction is also supported by one-hybrid assays using the ACS as a target (Dowell et al. 1994). These studies identified ScDbf4p as a factor that associates with an ACS-recognizing complex. A more detailed understanding of the interaction between ORC and other pre-RC components will only be possible through the characterization of assays for pre-RC formation using purified proteins.

The ORC ATPase at the origin: clamp loader or initiation detector

The ATP-dependence of ORC's interaction with Cdc6p and potentially other replication factors suggests that hydrolysis of ATP by ORC could have significant consequences for the formation, activation, or disassembly of the pre-RC (Fig. 3). One intriguing possibility is suggested by the similarity between several ORC subunits (Orc1p, 4p, and 5p), Cdc6p, and the subunits of the eukaryotic and prokaryotic clamp loaders (RF-C and the γ complex, respectively; Perkins and Diffley 1998). Studies of the clamp loading complexes indicate that they require binding to ATP to associate with and open the sliding clamp (e.g., PCNA). When the clamp loader associates with DNA, ATP hydrolysis is stimulated, causing the release of the sliding clamp such that it encircles the bound DNA (for review, see Ellison and Stillman 2001). A similar mechanism could be used by ORC and/or Cdc6p to assemble the ring-shaped MCM complex onto the origin during pre-RC formation (Fig. 3A; Adachi et al. 1997; Perkins and Diffley 1998). Consistent with this hypothesis, studies of an in vitro pre-RC assembly reaction indicate that ATP hydrolysis is required to assemble the MCM proteins onto the origin (Seki and Diffley 2000). Determining the proteins responsible for this ATPase activity is difficult due to the multiple ATPases involved in pre-RC formation. ORC, Cdc6p, and the MCM proteins are each known to hydrolyze ATP and it remains possible that there are other unknown ATPases involved in pre-RC formation. If the MCM proteins are loaded around the DNA during pre-RC formation they are most likely placed around a double stranded form of the origin. Studies of pre-RC complexes have found no evidence for the formation of unwound DNA (Geraghty et al. 2000; Walter and Newport 2000). Assuming that the MCM proteins act as the replicative DNA helicase (for review, see Labib and Diffley 2001), encircling dsDNA would be unusual as replicative DNA helicases are typically found to encircle ssDNA (e.g., see O'Donnell and Hingorani 2000 and references therein).

An alternative role for ATP hydrolysis by ORC is suggested by the ability of ssDNA to stimulate the ORC ATPase (Fig. 3B). It is possible that the formation of

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Figure 3. Two models for the ATP regulation of origin recognition complex (ORC). (A) Clamp loader. The similarity of ORC and Cdc6p to subunits of the RF-C and γ -complex clamp loaders has led to the suggestion that ORC/Cdc6p may act as a clamp loader for the ring shaped Mcm2-7p complex (MCM complex) during pre-RC formation. If so, it is likely that ORC/Cdc6p ATP binding is coupled to ring-opening of the MCM complex and that ATP hydrolysis is coupled to release and closure of the MCM complex ring around double-stranded DNA. (B) Initiation to elongation switch. An alternative hypothesis is that ATP hydrolysis by ORC is coupled to the dismantling of contacts made during pre-RC formation allowing a transition from an initiating state to an elongating state. If so, it is predicted that single-stranded DNA generated during initiation (ssDNA is known to stimulate the ORC ATPase and alter ORC conformation) alters the association of ORC with other replication factors allowing them to move away from the origin as part of the replication fork.

ssDNA at the origin during initiation triggers ORC ATP hydrolysis and a change in ORC conformation. These changes could then be coupled to restructuring of the origin associated replication machinery from a preinitiation/initiation conformation to an elongating conformation. Such a mechanism would be analogous to the changes in the associated protein complexes that occur at transcriptional promoters during the transition from initiation to the elongation (Conaway et al. 2000). If true, this mechanism would represent a robust method to ensure that the means to initiate DNA replication would not be lost until substantial ssDNA had been generated (the optimal length to stimulate the ORC ATPase is 80 bp).

These two proposed mechanisms for the roles of the ORC ATPase are not mutually exclusive. It remains possible that the ssDNA-stimulated activation of the ORC ATPase could contribute to the loading of the MCM

complex around ssDNA at the time of initiation. Indeed, the ssDNA stimulation of the ORC ATPase would be analogous to the DNA stimulation of ATP hydrolysis of the clamp loader. In both cases the formation/association with the nucleic acid target would stimulate the release and deposition of the ring shaped complex (i.e., the sliding clamp or the MCM complex).

Does ORC function after pre-RC recruitment?

What is ORC's role after pre-RC assembly? Studies in replicating *Xenopus* extracts indicate that ORC and Cdc6p can be removed from chromatin after pre-RC formation has occurred in G₁, and the remaining MCM proteins are competent to direct the initiation of DNA replication after S-phase entry (Hua et al. 1997; Rowles et al. 1999). This finding argues strongly that ORC is not required to recruit many of the components that assemble at the origin in an MCM-dependent manner. Such factors would include Cdc45p, RPA, and the eukaryotic DNA polymerases. In addition, these findings argue against a direct role in the DNA unwinding step of the initiation reaction, which is thought to occur after MCM loading and only on entry into S phase.

Despite the implied lack of function after pre-RC formation in *Xenopus* embryonic extracts, ORC ordinarily remains at the origin throughout the G₁ and S phases of the cell cycle (Rowles et al. 1996). Given that ORC remains at the origin during initiation, what could its function be? One possibility is that ORC remains merely to act as a chromosomal landmark to identify the site of each origin in subsequent cell cycles. For example, it is possible that ORC bound to one daughter chromosome could facilitate binding of ORC to the other daughter chromosome. Indeed, because the exact number of ORC complexes bound at each origin is unknown and could be greater than one (Wilmes and Bell 2002), there is a possibility that the retention of ORC at the origin could facilitate binding of additional ORC molecules at that same site. It is also possible that the artificial means of ORC removal used in the *Xenopus* extract experiments (e.g., salt extraction) actually reflects the loss of ORC-association with other replication factors at the origins that is ordinarily regulated by other means (e.g., by ATP-regulated changes, see above). Finally, the retention of ORC at origins could be involved in other nonessential functions not assayed in the *Xenopus* replication extracts (e.g., formation of heterochromatin, see below).

Regulation of ORC: subunit modification and complex remodeling

The central role of ORC function during the initiation of replication makes it a prime target for cell cycle regulators. In *S. cerevisiae*, the synthesis of ORC is not highly regulated either at a transcriptional or translational level. There is evidence, however, that ScORC is phosphorylated in a cell cycle-regulated manner on both the Orc2p and Orc6p subunits. Both biochemical and genetic

studies strongly suggest that this phosphorylation is dependent on cyclin-dependent kinases (Nguyen et al. 2001; Vas et al. 2001). Strains lacking the consensus CDK phosphorylation sites on Orc2p and Orc6p are found to replicate normally, indicating that phosphorylation of these sites is not essential for the initiation of replication. Instead, it appears that these modifications to ScORC inhibit new pre-RC formation after entry into S phase. In *S. cerevisiae*, the simultaneous mutation of the CDK phosphorylation sites on ORC, elimination of the Cdc6p N-terminus (preventing its degradation), and constitutive localization of MCM proteins to the nucleus result in a strain that inappropriately reinitiates DNA replication within the same cell cycle, presumably by allowing new pre-RC formation (Nguyen et al. 2001). Similar studies of *S. pombe* ORC also suggest that phosphorylation of ORC negatively regulates reinitiation of replication (Vas et al. 2001).

The molecular consequences of ORC phosphorylation that inhibit pre-RC formation are unclear. The simplest hypothesis is that modification directly inhibits association between ORC and other components of the pre-RC. Alternatively, phosphorylation could alter an activity of ORC that is indirectly required for pre-RC formation (e.g., ATP binding) although no effect of phosphorylation on ORC's biochemical activities has been described. A better understanding of the consequences of such modifications will be obtained through the study of reconstituted assays for pre-RC formation from both *Xenopus* and yeast. These studies have already demonstrated that the addition of CDK activity or S phase extract inhibits new pre-RC formation (e.g., see Seki and Diffley 2000) and further dissection of these assays should provide a molecular understanding of the inhibitory mechanisms.

The regulation of ORC function in metazoans is likely to include other mechanisms in addition to phosphorylation. Unlike the relatively stable expression of ORC in yeast cells, studies of *DmORC1* suggest that this subunit is limiting for DNA replication (Asano and Wharton 1999). In addition, in both mammalian and *Drosophila* cells *ORC1* transcription is responsive to E2F1 and is regulated during the cell cycle (Ohtani et al. 1996; Asano and Wharton 1999). A second mechanism to regulate ORC function in mammalian cells may involve alteration of the association of ORC subunits. Unlike ORC from budding yeast, *Drosophila*, and *Xenopus*, the subunits of the SpORC and mammalian ORC are not always found in a stable complex (Moon et al. 1999; Natale et al. 2000; Thome et al. 2000). For example, SpOrc4p is retained on chromatin under conditions that elute the remainder of SpORC (Moon et al. 1999). Similarly, whereas mammalian Orc2p is found constitutively on the chromatin, mammalian Orc1p is removed from the chromatin at the end of S phase and only rebinds as cells reenter G₁ (Natale et al. 2000; Tatsumi et al. 2000; Kreitz et al. 2001). The mechanisms that control the association of the ORC subunits are unclear but may include proteolysis. Recent studies suggest that HsOrc1 is proteolyzed during S phase as a mechanism to prevent re-replication (Kreitz et al. 2001). In contrast, other studies

have found HsORC1p is stable throughout the cell cycle (Saha et al. 1998; Okuno et al. 2001; Li and DePamphilis 2002). In an interesting cross section of these apparently contradictory results, a recent study has demonstrated that ORC derived from Hamster cells is stable during the cell cycle but differentially modified by a limited number of ubiquitin conjugates during the cell cycle (Li and DePamphilis 2002). It is possible that the differences reflect different mechanisms of mammalian Orc1p regulation in different cell lines/types.

Consistent with the existence of HsORC lacking Orc1p, studies addressing the assembly of HsORC found that a core subcomplex of HsOrc2-5p was readily assembled in insect cells (Dhar et al. 2001a; Vashee et al. 2001), but that a six subunit complex was only isolated if the nuclei of the same cells are extracted with high salt (Vashee et al. 2001). Together, these findings suggest that the six subunit HsORC is only assembled on the chromatin during the G₁ and S phase of the cell cycle. If true, this model suggests that a complex of HsORC lacking HsOrc1p is competent for chromatin binding (unlike the situation in *Drosophila* and *S. cerevisiae*), although the specificity of this interaction is unclear.

ORC: not just for replication anymore

ORC's functions extend beyond DNA replication. As described above, not all of the ORC binding sites in the *S. cerevisiae* cells colocalize with origins of replication. Interestingly, the majority of these sites colocalize heterochromatin, including telomeres and one of the silent mating type loci (*HML*). In addition, ORC has been implicated in the control of M phase events, including chromosome condensation and integrity.

ORC and the establishment of heterochromatin

The clearest case of an alternate function for ORC is in the establishment of transcriptionally repressed domains at the *S. cerevisiae* silent mating type loci, *HMR* and *HML* (for review, see Shore 2001). ORC's role in silencing was recognized early on through genetic screens that identified mutations in *ScORC2* and *ScORC5* as defective for mating type silencing (Foss et al. 1993; Micklem et al. 1993). The role of ORC in silencing was not surprising as all four of the mating-type silencers (*HMRE*, *HMRI*, *HMLE*, and *HMLI*) were known to contain matches to the ACS and act as replication origins on plasmids (Dubey et al. 1991). Subsequent genetic studies demonstrated that mutants of ORC genes could be identified that distinguished between ORC's function in DNA replication and its role in transcriptional silencing (Bell et al. 1995; Fox et al. 1995; Dillin and Rine 1997). Cloning of the *ORC1* gene found that it encoded a protein with substantial similarity to Sir3p, an essential factor for mating-type silencing. This similarity was particularly strong at the N terminus and a mutation that removes this domain results in a loss of transcriptional silencing but has no effect on DNA replication (Bell et al.

1995). The role of this domain in mating-type silencing is to recruit Sir1p, a second key protein in the establishment of mating-type silencing (Triolo and Sternglanz 1996; Fox et al. 1997).

Although ORC clearly contains separable domains required only for silencing, it remained possible that ORC's role at the silencers also involves a DNA replication function. This hypothesis is supported by a number of different findings, including a seminal study indicating that passage through S phase is required for the establishment of silencing (Miller and Nasmyth 1984) and the identification of mutations in several other DNA replication genes as defective in silencing (Axelrod and Rine 1991; Ehrenhofer-Murray et al. 1999). In addition, each of the cis-acting sequences required for mating-type silencing has the potential to act as an origin on a plasmid (Dubey et al. 1991). In contrast, other studies argue against a replicative role for ORC in silencing. For example, the mating-type silencers associated with the *HML* locus fail to initiate replication at their normal chromosomal position (Dubey et al. 1991). In addition, alleles of ORC have been identified that are defective in replication but not silencing (Dillin and Rine 1997). Recent studies strongly support the latter hypothesis. Using altered silencers that have replaced ORC and the ACS with Gal4p–Sir1p fusion protein and Gal4 DNA binding sites respectively, silencing could be established on episomes that lack origins and are therefore unable to replicate (Kirchmaier and Rine 2001; Li et al. 2001). Thus, neither the generation nor the passage of replication forks is required for the establishment of these silenced loci. Even under these circumstances, however, the requirement for passage through S phase is retained.

A function in the establishment of transcriptionally repressed regions may be a conserved feature of ORC. Several lines of evidence support a role for DmORC in the establishment or maintenance of the *Drosophila* heterochromatin (Pak et al. 1997; Huang et al. 1998). First, DmORC interacts directly with HP1, a key component of *Drosophila* heterochromatin, and a similar interaction is also observed between XlORC and *Xenopus* HP1. In vitro studies localized the strongest interaction domain to the N terminus of DmOrc1p (Pak et al. 1997), similar to the requirement of the N terminus of ScOrc1p to interact with Sir1p. This region overlaps a bromo-adjacent homology (BAH) domain that is found in multiple Orc1 proteins as well as Sir3p and DNA methyl-transferases, although the functional significance of this domain is unknown (Callebaut et al. 1999). Second, mutations in DmORC result in mislocalization of HP1, suggesting that, as for *S. cerevisiae* Sir1p, DmORC acts to recruit HP1 to heterochromatin. Consistent with this hypothesis, DmORC is enriched at heterochromatic regions of the *Drosophila* genome (Pak et al. 1997). Third, mutations in DmORC suppress heterochromatin-dependent transcriptional repression (i.e., position-effect variegation) (Pak et al. 1997).

Together these findings support the hypothesis that ORC is involved not only in the replication of chromosomes but also in the establishment and maintenance of

at least a subset of chromatin domains. The reason to use ORC as opposed to any of hundreds of other DNA binding proteins is unclear. It is possible that ORC has been singled out because of its regular distribution along the chromosome (Wyrick et al. 2001). Alternatively, ORC may play a role in regulating chromatin structure that is yet to be fully appreciated. Along these lines, it has been demonstrated that ORC is responsible for positioning nucleosomes at *ARS1* (Lipford and Bell 2001). In addition, HsORC interacts with the histone acetyl transferase, HBO1 (Iizuka and Stillman 1999; Burke et al. 2001). Finally, despite the fact that replication fork passage is not required for the establishment of silencing, it is possible that the tight coupling between DNA replication and chromatin assembly may have initially favored proteins associated with origins to recruit chromatin assembly factors required for heterochromatin assembly.

Does ORC function in M phase?

The coupling of DNA replication to chromatid cohesion and chromatin assembly (for reviews, see Skibbens 2000; Mello and Almouzni 2001) raises the possibility that ORC could be involved in either of these events or other events that may depend on these functions. Genetic analysis of ORC mutant cells has found that these cells have defects in passage through M phase. Despite this, more recent studies suggest that these phenotypes are most likely caused by secondary consequences of replication defects and not a direct involvement of ORC in these events.

Many temperature sensitive ORC mutants in *S. cerevisiae* arrest prior to mitosis with at least a proportion of the cells having largely completed DNA replication (Bell et al. 1993; Dillin and Rine 1998). Although it was possible that these alleles were incompletely penetrant (it is likely as few as one in five origins must be activated to replicate the *S. cerevisiae* genome), these findings also raised the possibility that a loss of ORC function prevented some event in M phase. Interestingly, this arrest was still observed when the DNA replication/damage checkpoint proteins Rad9p or Mec1p were mutated (Dillin and Rine 1998), suggesting that the G₂ arrest was not caused by checkpoint pathways involving these proteins. In contrast, if the mutant cells were arrested at M phase and then brought to the nonpermissive temperature and released, cells arrested with a G₁ DNA content. This suggested that ORC mutants could arrest at either G₂/M or prior to the initiation of DNA synthesis. Similar findings have been observed in *Drosophila*. Flies carrying homozygous mutations of *DmORC2* and *DmORC5* show clear defects in DNA replication but are also defective in chromosome condensation, have an elevated levels of chromosome breakage, and an abnormally high number of cells arrested in metaphase (Loupert et al. 2000; Pflumm and Botchan 2001).

Does ORC function directly in M phase? Because these experiments only assessed the final arrest point of the ORC mutations, it remained possible that the failure to complete M phase was attributable to defects in DNA

replication inducing other checkpoint mechanisms rather than a direct role of ORC in mitosis. Consistent with this hypothesis, determination of the cell cycle execution point of ScORC using multiple ORC mutations identified a narrow window spanning late G₁ phase through early S phase during which ORC is required for normal cell cycle progression (O. Aparicio and S.P. Bell, unpubl.). Because the alleles tested are known to eliminate ORC DNA binding (Aparicio et al. 1997), this finding strongly suggests that there is no requirement for chromatin bound ORC from early S phase to the completion of M phase for cell cycle progression. Similarly, immunofluorescence studies of XlORC in *X. laevis* tissue culture cells suggest that ORC is removed from the chromatin during M phase (Romanowski et al. 1996).

What is responsible for the G₂/M arrest observed in ScORC subunit mutations? Further studies of these alleles suggest that they activate the spindle assembly checkpoint leading to the G₂/M arrest (O. Aparicio, unpubl.). Although the mechanism for the activation of the checkpoint remains unclear, it is likely that incomplete replication of the chromosomes results in defects in spindle assembly. Interestingly, mutation of Cdc13p, a protein involved in the duplication of telomeres, also results in the activation of the spindle assembly checkpoint (Wang et al. 2000).

It is likely that the mitotic defects observed in DmORC mutations are also the indirect result of DNA replication defects. The phenotypes of the *Drosophila* ORC mutations are similar to those of other mutations in DNA replication proteins including mutant alleles of *DmMCM4* and *DmPCNA* (Pflumm and Botchan 2001). In addition, sites of altered chromosome condensation appear to correlate with sites that exhibit altered replication timing in embryos mutant for *DmORC2*. Thus, in both *S. cerevisiae* and *Drosophila*, a general defect in DNA replication may have consequences for the correct preparation of chromosomes for the subsequent mitosis. Although it is unclear how these alterations in DNA replication lead to defects in chromosome condensation, these findings suggest that there are important events that must be coordinated between DNA replication and mitosis.

Studies of the localization of the Orc6 in *Drosophila* and human cells suggest a more direct connection between ORC and other events of the cell cycle. Studies in *Drosophila* cells indicate that there is a substantial excess of DmOrc6p relative to the other DmORC subunits. This protein is primarily in the cytoplasm and is particularly concentrated at the cell membrane (Chesnokov et al. 2001). Interestingly, observation of DmOrc6 localization during the end of mitosis indicates that it is concentrated at the cytokinetic furrow (I. Chesnokov and M. Botchan, unpubl.). In human cells, HsOrc6p also appears to be localized at the cytokinetic furrow as well as at the kinetochore (B. Stillman, unpubl.). Although the exact function of this localization remains to be determined, it is intriguing to speculate that metazoan Orc6p is involved in a feedback mechanism prevent-

ing ORC function or pre-RC formation until mitosis is completed.

Conclusions

The identification of ORC has led the way in the characterization of the proteins that assemble at origins during the cell cycle. By studying these assembly events, many of the key mechanisms controlling DNA replication during the cell cycle have been determined. Despite a detailed knowledge of the order and timing of replication factor association with the origin, our understanding of the biochemical function performed by these factors remains primitive for the majority of the initiation factors, and ORC is no exception. Although it is clear that ORC associates with specific regions of the genome, the mechanism by which it arrives at these sites remains obscure, particularly in metazoans. Similarly, the role of ORC in the formation of the pre-RC and the control of ORC activity by ATP binding and hydrolysis remain to be determined. Both new biochemical assays and additional structural studies of ORC will be essential to advance our understanding of this critical complex.

The function of ORC outside of DNA replication represents just one of an increasing number of connections between different regulators of chromosome propagation. For example, recent studies indicate that correct sister chromatid cohesion influences DNA repair and recombination (Sjogren and Nasmyth 2001; Watanabe et al. 2001). The detection of ORC mutations by the spindle checkpoint suggests that there is coordination between DNA replication and the preparation of chromosomes for segregation that remains to be understood. Similarly, although the role of Orc6 at the cytokinetic furrow is unclear, such observations are likely to lead to unanticipated molecular connections. Indeed, as the events that control chromosome duplication and segregation are understood in mechanistic detail, it will become increasingly important to investigate how these events are coordinated with one another. The ability to identify the genome-wide location of the factors that control chromosome duplication and segregation will provide information about the chromosomal landscape and insights into the connections between them.

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